

Legends to Supplementary Figures

Figure S1. Pathway of coronary vessel sprouting and invasion. Migration pathway of coronary vessels as they sprout from the sinus venosus and spread over and invade the heart. Arrows show direction of new vessel growth, which occurs superficially (solid lines) or deep in the myocardial layer (dashed lines). Filled circles, blood island-like structures. Bottom panel shows location of coronary arteries (ca, red), veins (cv, blue), and capillaries (cap) in the adult.

Figure S2. Coronary vessel development visualized by CD31 staining. Whole mount embryonic mouse hearts of the indicated age stained with anti-CD31 antibody and peroxidase immunohistochemistry (brown). Note that CD31-positive endocardial cells (boxed and insets at e12.5) are difficult to distinguish from invading coronary sprouts (is, inset e13.5 ventral), both of which are deep to the coronary surface sprouts (ss, filled arrowheads) and intermingled with myocardial cells. Open arrowheads, blood island-like structures (bi); sv, sinus venosus; ra, right atria; rv, right ventricle; la, left atria; lv, left ventricle. Scale bars, 200 μm .

Figure S3. Specificity of apelin-nlacZ transgene expression in the embryonic heart. Section of e13.5 heart from apelin-nlacZ transgenic mice immunostained for nuclear β -galactosidase (nlacZ) expression (βgal , red) and endothelial/endocardial marker CD31 (green). Nuclei were labeled with DAPI (blue). Coronary vessel endothelial cells (cv) express both markers, whereas endocardial cells (endo) express only CD31. Inset is close-up of coronary vessels (boxed region). Although the subepicardial endothelial cells (apelin-nlacZ⁺, CD31⁺) are very close to the epicardium, we have not detected epicardial

($\alpha 4$ integrin⁺) cells expressing the apelin-lacZ marker (K.R.H. and M.K., unpublished data). epi, epicardium; myo, myocardium. Scale bar, 100 μ m.

Figure S4. Absence of VE-cadherin-expressing cells in the proepicardium. a.

Schematic of an e9.5 embryo with box indicating region shown in **b** and **c**. da, dorsal aorta; cv, cardinal vein; sv, sinus venosus. **b, c**. Sagittal sections (80 μ m) of e9.5 embryos immunostained for $\alpha 4$ integrin (green) to label proepicardium (pe) and for VE-cadherin (red) to label endothelial cells. **b', c'**. Close-up of boxed regions. The proepicardium does not contain endothelial cells but is directly adjacent to the developing liver plexus (arrowheads). Major heart structures (sv, sinus venosus; ra, right atria; rv, right ventricle; ot, outflow tract) are outlined. Scale bar, 100 μ m.

Figure S5. Lineage tracing with Tbx18-Cre shows that proepicardial cells do not contribute to the endothelium of the sinus venosus or coronary sprouts. a.

Sagittal section of an e10.5 Tbx18-Cre; RosaYFP reporter embryo immunostained for the endothelial cell marker CD31 (left panel and red in merged image in right panel) and for YFP (middle panel and green in merged image in right panel) to show cells derived from the proepicardium and other Tbx18-expressing cells. Nuclei are labeled with DAPI (blue in right panel). **b**. Close-up of sinus venosus (upper box in **a**). Note that none of the endothelial cells (arrowheads; CD31⁺, red) express the Tbx18-Cre lineage trace (YFP, green). **c**. Close up of a coronary sprout budding from the sinus venosus (lower box in **a**). Note that none of the endothelial cells (arrowheads; CD31⁺, red) express the Tbx18-Cre lineage trace (YFP, green). Out of more than 4100 sinus venosus and coronary sprout endothelial cells analyzed from seven e10.5 and e11.5 embryos, no cells expressing YFP

and CD31 were detected. cs, coronary sprout; liv, liver; ra, right atria; rv, right ventricle; sv, sinus venosus. Scale bar, 100 μ m.

Figure S6. Absence of VEGFR2⁺/CD31⁻ endothelial progenitors in the proepicardium and heart wall. Sagittal sections of developing embryos of the indicated ages immunostained for either VEGFR2 (red), CD31 (green), and CD34 (blue) (**a** and **c**) or VEGFR2 (red), CD31 (green), and Wt1 (blue) (**b**). (**a**) Close-up of area around developing dorsal aorta (da), a vessel that forms by vasculogenesis. Note that VEGFR2⁺/CD31⁻ endothelial progenitors (red, arrowheads) are abundant around the developing dorsal aorta. In 19 da sections from 4 embryos, we counted >380 VEGFR2⁺/CD31⁻ near >1500 da endothelial cells. **b.** Close-up showing region around the proepicardium (pe; Wt1⁺, blue). In 156 pe sections from 9 embryos ages e8.5-10.5, no VEGFR2⁺/CD31⁻ cells were detected. (The neighboring blood vessel (bv) contains many VEGFR2⁺/CD31⁺ cells, but no VEGFR2⁺/CD31⁻ cells.) **c, c'.** Close-up of coronary sprout (cs; white dots) emerging from the sinus venosus (sv) and detail (**c'**) of boxed region just ahead of the growing coronary sprout. Note that no VEGFR2⁺/CD31⁻ cells are detected, including the region just ahead of the growing coronary sprout (arrowheads). VEGFR2⁺/CD31⁻ cells are absent or extremely rare in the heart wall: in 82 heart wall sections from 8 embryos ages e11.5 and 12.5, we detected only two such cells near the over 5000 coronary sprout endothelial cells analyzed. Scale bars, 50 μ m (**a** and **b**) and 100 μ m (**c**). endo, endocardium; ven, ventricle; ra, right atria; rv, right ventricle.

Figure S7. Spatial distribution of clones in Table S1. Schematics of each clone as in Figure 3, with marked cells in the clone highlighted in purple. Clone identification

numbers from Table S1 are indicated. Only representative examples of Type V and VIII clones are shown. VI and VII are not shown.

Figure S8. Downregulation of Coup-TF2 expression in sinus venosus sprouts.

Adjacent tissue sections of e12.5 heart immunostained for CD31 (left panel) to mark endothelial cells and hybridized with a COUP-TFII RNA probe (right panel) to show COUP-TFII expression. COUP-TFII is expressed in cells lining the sinus venosus (sv, filled arrowheads), but expression is downregulated as sinus venosus sprout (svs, open arrowheads) migrates away from the sinus venosus to form coronary vessels. Scale bar, 200 μ m.

Figure S9. Expression of arterial and venous markers in the embryonic heart.

Immunostains (**a, b, j, k, j'-s'**) and in situ hybridization (**c-i, l-s**) of sagittal sections of hearts from wild type (**a, c-i, l-s**), ephrinB2-lacZ (**b** and **j**) or EphB4-lacZ (**k**) embryos of the indicated ages showing expression of the indicated markers. **l'-s'**, adjacent sections to ones shown above in **l-s**. CD31 staining shows distribution of endothelial and endocardial cells. Note three layers in cardiac wall: a superficial, subepicardial layer (SE, blue line), a deeper layer within the myocardium (M, red line), and an inner endocardial layer (En, black line) that lines the cardiac chamber. Asterisks show layers with detectable expression of the marker: asterisks denote high expression and asterisks in parentheses indicate low expression. **a-i**. At e13.5, ephrinB2 and other arterial markers (red) are preferentially expressed in the myocardial layer whereas venous markers (blue) are preferentially expressed in the subepicardial layer. The only exception is the arterial marker Notch4, which, at this stage, is expressed at similar levels in both layers. **j-s**. At e15.5, selective expression of arterial and venous markers in the two layers is more pronounced with

arterial markers expressed exclusively in endothelial cells in the myocardial layer and venous markers expressed exclusively in endothelial cells in the subepicardial layer. Red arrowheads, coronary arteries; blue arrowheads, coronary veins. Scale bar (for a-s), 50 μ m.

Figure S10. Key sites of regulation during coronary artery development. Outgrowth and invasion pathway of sinus venosus sprouts (blue line) as they undergo venous dedifferentiation (black) and transdifferentiation/redifferentiation into coronary arteries (red) and veins (blue). Sites in the heart where the endothelial cell transitions occur are highlighted (dashed ovals) along with the types of developmental signals that are presumably present at these sites to induce the specific transitions.

Movie S1. Timelapse video (10 frames/sec) of intact heart cultured for 72 hours.

Movie S2. Timelapse video (10 frames/sec) of dissected ventricle cultured for 72 hours.

Movie S3. Timelapse video (10 frames/sec) of dissected atria cultured for 72 hours.

Movie S4. Timelapse video (10 frames/sec) of recombined heart cultured for 72 hours.